REMARKS

Amendments

Claims 1, 6, 7, 16, 17, 18, 27, and 74-78 have been amended to recite that the dominant negative mismatch repair protein is a dominant negative PMS2-134 protein. Claims 72 and 73 have been amended to recite "a dominant negative PMSR3 mismatch repair protein" in place of "a dominant negative mismatch repair protein selected from the group consisting of a dominant negative PMSR and a dominant negative PMS2L mismatch repair protein." The specification supports these amendments at the disclosure that "the findings disclosed here teach the use of MMR genes, including the human PMSR2 and PMSR3 gene, the related PMS134 truncated MMR gene, the plant mismatch repair genes and those genes that are homologous to the 134 N-terminal amino acids of the PMS2 gene that include the MutL family if MMR proteins and including the PMSR and PMS2L homologs described by Hori et al. and Nicolaides to create hypermutable microbes." Page 18, lines 8-16, citations omitted.

The amendments introduce no new matter.

The Rejection of Claims 1, 6, 16-18, 26, 27, and 71-78 Under 35 U.S.C. § 112

Claims 1, 6, 7, 16-18, 26, 27, and 71-78 stand rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the written description requirement. Applicants respectfully traverse.

Claims 1, 18, 72, and 73 are the independent claims of the rejected claim set. Claim 1 and 72 are directed to methods for making a hypermutable bacterium. A polynucleotide encoding a dominant negative mismatch repair protein under the control of an inducible transcription regulatory sequence is introduced into a bacterium. The inducible transcription regulatory sequence in the bacterium is induced. The dominant negative mismatch repair protein exerts a dominant negative effect on mismatch repair when expressed in the bacterium. The bacterium becomes hypermutable. Claims 18 and 73 are directed to homogenous compositions of induced, cultured, hypermutable bacteria which comprise a polynucleotide encoding a dominant negative mismatch repair protein under the control of an inducible transcription regulatory sequence. The dominant negative mismatch repair protein exerts a dominant negative effect when expressed in the bacteria. Claims 1 and 18 recite that the dominant negative mismatch repair protein is a dominant negative PMS2-134 mismatch

repair protein. Claims 72 and 73 recite that the dominant negative mismatch repair protein is a dominant negative PMS3R mismatch repair protein.

Compliance with the written description requirement of 35 U.S.C. § 112, first paragraph requires sufficient information in the original disclosure to convince an ordinarily skilled artisan that the inventor possessed the invention at the time of filing. *Moba, B.V. v. Diamond Automation, Inc.*, 325 F.3d 1306 (Fed. Cir. 2003). If the claimed invention recites a genus, the written description requirement for the claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, *i.e.*, structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus. *Regents of the University of California v. Eli Lilly*, 119 F.3d 1559 (Fed. Cir. 1997).

The Advisory Action maintains that the claims are not adequately described because the specification and the art do not describe a representative number of mismatch repair proteins which when expressed in any bacterium will exert a dominant negative effect resulting in a hypermutability. Advisory Action dated June 7, 2005 at page 2, lines 14-21.

To advance prosecution, the claims have been amended to recite that in the methods for making a hypermutable bacterium and the homogeneous compositions of induced, cultured, hypermutable bacteria employ a polynucleotide encoding a dominant negative PMS2-134 or PMSR3 mismatch repair protein. The specification describes these genera of dominant negative mismatch repair proteins.

The specification describes the genera of PMS2-134 and PMSR3 mismatch repair proteins by providing examples of the claimed methods of making a hypermutable bacterium and homogeneous compositions of induced, cultured, hypermutable bacteria using polynucleotides encoding a representative number of species of the genus of dominant negative mismatch repair proteins. The specification discloses, in Examples 2 and 3, that expression of human PMS2-134, *Arabidopsis thaliana* PMS2-134, or human PMSR3 in various strains of *E. coli* cells induces hypermutability. See Example 2 at page 26, line 14 to page 27, line 4 and Table 1; See Example 3 at page 29, lines 4-7 and Table 2. Thus, the specification describes hypermutability as a phenotype resulting from expression of two species of dominant negative PMS2-134 mismatch repair proteins, human and *A. thaliana*

PMS2-134, and one species of dominant negative PMSR3 mismatch repair protein, human PMSR3.

Applicants also satisfy the written description requirement for the recited genus of polynucleotides encoding dominant negative mismatch repair proteins through sufficient description of a representative number of species of the encoded proteins by identifying characteristics, *i.e.*, structure. The specification discloses that PMS2-134 truncated proteins and PMSR3 proteins are highly homologous, *i.e.*, share a similar structure. The specification discloses that "the use of MMR genes, including the human PMSR2 and PMSR3 gene (ref 19), the related PMS134 truncated MMR gene (ref 32), the plant mismatch repair genes and those *genes that are homologous* to the 134 N-terminal amino acids of the PMS2 gene ... [are useful] to create hypermutable microbes." Page 18, lines 8-16, emphasis added. Thus, the specification discloses that the species encompassed by the recited genus of dominant negative PMS2-134 and PMSR3 mismatch repair proteins share a common structural feature. Based on the description in the specification of these identifying structural characteristics of the recited genus of dominant negative mismatch repair proteins and the specific exemplification of hypermutable bacteria produced by expression of a representative number of species of the recited genus of dominant negative mismatch repair proteins, one of skill in the art would have understood that applicants possessed the claimed invention at the time of filing.

The specification also adequately describes that expression of the recited genera of PMS2-134 and PMSR3 proteins will confer hypermutability in *any* bacteria. The specification discloses,

> The term bacteria used in this application includes any organism for the prokaryotic *kingdom.* These organisms include genera such as but not limited to *Agrobacterium*, *Anaerobacter*, *Aquabacterium*, *Azorhizobium*, *Bacillus*, *Bradyrhizobium*, *Cryobacterium*, *Escherichia*, *Enterococcus*, *Heliobacterium*, *Klebsiella*, *Lactobacillus*, *Methanococcus*, *Methanothermobacter*, *Micrococcus*, *Mycobacterium*, *Oceanomonas*, *Pseudomonas*, *Rhizobium*, *Staphylococcus*, *Streptococcus*, *Streptomyces*, *Thermusaquaticus*, *Thermaerobacter*, *Thermobacillus*, etc. Other procaryotes that can be used for this application are listed at the website having the URL address www host server, bacterio.cict.fr domain name, validgenericnames directory.

Page 12, lines 7-16. Nonetheless, the Advisory Action asserts that

> There is no support in the specification or in the prior art that said truncations will confer hypermutability in all species of bacterium (e.g., gram positive). For example, to date, there is no structure-function relationship that has been identified in gram-positive bacteria for MutL (i.e., PMS2) homologues. If said relationship is unknown for PMS2 homologues in gram-positive bacteria, it logically follows that results obtained for PMS2-134 are not necessarily extendable to all species of bacteria (e.g., expression of PMS2-134 in gram positive).

Advisory Action at page 2, line 18 to page 3, line 3. As pointed out above, the specification teaches that the genera of dominant negative mismatch repair proteins will induce hypermutability in all types of bacteria, including gram positive bacteria. Furthermore and contrary to the assertion in the Office Action, the art, to date, does teach that gram-positive bacteria express MutL homologues (like PMS2) having similar structures, *e.g.*, sequences, and functions. See Exhibits A-D.[1]

Mankovich *et al.* (*J. Bacteriol. 171* (1989):5325-31; Exhibit A), Merino *et al.* (*Mol. Microbiol. 44* (2002):877-87; Exhibit C), and Ginetti *et al.* (*Microbiology 142* (1996):2021-9; Exhibit D) each teach a MutL protein of a Gram positive bacteria that has a similar structure; each is taught to have a structure similar to that of *Streptococcus pneumoniae* HexB.

- Mankovich at lines 3-5 of the abstract: "MutL [of *Salmonella typhimurium*] was found to be quite similar to the HexB mismatch repair protein of S. pneumoniae ... The significant similarities among these proteins were confined to their amino-terminal regions." *S. pneumoniae* is a Gram positive bacteria.
- Merino at lines 7-10 of the abstract: "The deduced ... MutL [protein of *L. monocytogenes*] shares 59% identity with MutL of B. subtilis and 47% identity with HexB of S. pneumoniae." Lines 7-10 of the abstract. *L. monocytogenes* and *B. subtilis* are Gram positive bacteria
- Ginetti at lines 5-6 of the abstract: "The deduced [B. subtilis] MutS protein is 49% identical to HexA and MutL is 46% identical to HexB of Streptococcus pneumoniae." *B. subtilis* is a Gram positive bacteria

In addition, *E. coli* (a Gram negative bacteria) MutL has been established to have homology to *S. pneumoniae* HexB. See Prudhomme (*J. Bacteriol. 173* (1991):7196-7203), cited by the

[1] Exhibits A-D include publications from both before and after applicants' effective filing date, February 11, 2000. Applicants' citation of pre- and post-filing evidence refutes the Patent Office's doubt regarding operability across the full scope of the claims. Particularly, the Patent Office doubted that the claimed mismatch repair proteins would induce hypermutability in all strains of bacteria. Use of post-filing date evidence is appropriate for this purpose. See *In re Marzocchi and Horton*, 169 USPQ 367, 370 (CCPA 1971).

Patent Office and discussed below. Thus, the structure of MutL homologues in both Gram positive and Gram negative bacteria is similar.

Moreover, the claimed dominant negative mismatch repair proteins would be predicted to affect the process of mismatch repair in Gram positive bacteria similar to that in Gram negative bacteria as demonstrated by the following teaches in the art. Mankovich, Merino, Ginetti, and Prunier *et al.* (*J. Bacteriol. 187* (2005):3455-64; Exhibit B) teach that the function of MutL homologues in Gram positive bacteria is similar; they function in mismatch repair and can induce hypermutable phenotypes.

Mankovich teaches that overexpression of *S. typhimurium* MutL in *S. typhimurium* (a Gram positive bacteria) results in a hypermutable phenotype: "The assignment of reading frame was confirmed by the construction of a chimeric protein consisting of the first 30 amino acids of LacZ fused to residues 53 through 618 of MutL. Interestingly, the presence of excess amounts of this fusion protein in wild-type mutL+ cells resulted in a trans-dominant effect causing the cell to exhibit a high spontaneous mutation frequency." Lines 7-10 of the abstract.

Prunier teaches that inactivating or overexpressing *Staphylococcus aureus* MutL protein in *S. aureus* (a Gram positive bacteria) induces hypermutability. Prunier teaches, "Insertional inactivation of mutS and mutL genes and complementation showed the role of both genes in hypermutability in this species." Lines 6-7 of the abstract. Prunier further teaches "In four of five isolates with mutated or deleted mutS or mutL genes, a relationship between alterations and mutator phenotypes could be established by negative complementation of the mutS or mutL mutants." Lines 11-13.

Merino teaches that deletion of the *L. monocytogenes* (a Gram positive bacteria) MutL and MutS proteins results in a hypermutable phenotype. Merino teaches, "Functional analysis of the mutSL locus was studied by constructing a double knock-out mutant. We showed that the deletion DeltamutSL induces: (i) a 100- to 1000-fold increase in the spontaneous mutation rate; and (ii) a 10- to 15-fold increase in the frequency of transduction, thus demonstrating the role of mutSL of L. monocytogenes in both mismatch repair and homologous recombination. Lines 10-15 of the abstract.

Ginetti teaches that deletion of *B. subtilis* (a Gram positive bacteria) MutL and MutS proteins results in a hypermutable phenotype. Ginetti teaches, "Deletion of both mutS and mutL [in *B. subtilis*] resulted in an increase in the frequency of spontaneous mutations and abolished the marker effect observed in transformation." Lines 3-5 of the abstract.

Thus, the art teaches that not only do gram positive bacteria have a similar structure, but also a similar function. The art refutes the Patent Office's position that there is no structure-function relationship identified for MutL homologues in Gram positive bacteria.

The Advisory Action also maintains that references Prudhomme, Kondo, and Nicolaides support the rejection of the claims as not adequately described. Prudhomme teaches that expression of a *Streptococcus pneumoniae* homologue of the *Escherichia coli* MutL protein, HexB, in *E. coli* does not increase the mutation rate of *E. coli*. Prudhomme, thus, teaches that a protein (HexB of *S. pneumoniae*) having a structure analogous to full-length PMS2 does not increase the mutation rate of *E. coli*. None of the pending claims, as amended, recite a dominant negative mismatch repair protein having a structure analogous to full-length PMS2. Claims 1 and 18 are directed to methods for making a hypermutable bacterium and composition of induced, cultured, hypermutable bacteria that employ a polynucleotide encoding "a dominant negative PMS2-134 mismatch repair protein" (claim 1) or a "dominant negative mismatch repair protein [which] is PMS2-134" (claim 18). Claims 72 and 73 are similar to claims 1 and 18, but recite that the dominant negative mismatch repair protein is "a dominant negative PMSR3 mismatch repair protein." The specification clearly discloses that PMSR3 has a structure similar to that of PMS2-134. See page 18, lines 8-16, quoted above. Prudhomme therefore does not teach that a protein having a structure similar to that recited in the pending claims does not confer hypermutability in bacterial cells. Thus, Prudhomme is not evidence that one of skill in the art would doubt that he could predict that the genera of proteins encompassing PMS2-134 or PMSR3 induce hypermutability.

The Patent Office cites the Kondo reference in support of its position that the specification does not adequately describe the genus of dominant negative PMS2L proteins. The claims have been amended to no longer encompass polynucleotides encoding dominant negative PMS2L mismatch repair proteins. Thus, the Kondo reference is no longer applicable to the pending claims.

The Patent Office has cited the Nicolaides reference as teaching that "the function of PMSR genes is not definitively known" (final Office Action at page 5, lines 9-10) and that although applicants demonstrate that hPMSR3 induces hypermutability in bacterial cells, it "would not necessarily be predictive to other proteins having the prescribed functionality" (final Office Action dated December 23, 2004 at page 5 lines 11-12). The claims have been amended to recite polynucleotides encoding dominant negative PMSR3 mismatch repair

proteins in place of dominant negative PMSR mismatch repair proteins. The specification discloses that expression of hPMSR3 induces hypermutability in bacterial cells. This provides a definitive function for the genus of PMSR3 mismatch repair proteins and describes the genus of dominant negative PMSR3 mismatch repair proteins.

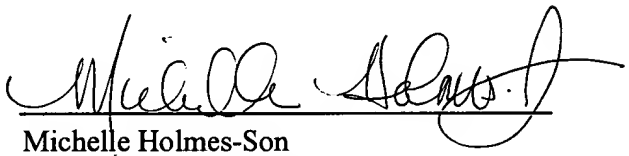
Applicants therefore respectfully submit that the Prudhomme, Kondo, and Nicolaides references provide no reason to doubt that substitution of any species of PMS2-134 or PMSR3 protein would function as a dominant negative mismatch repair protein in bacteria. Furthermore, applicants' specification provides adequate supporting disclosure for the claimed methods for making hypermutable bacteria and compositions of hypermutable bacteria that comprise a polynucleotide encoding a dominant negative PMS2-134 or PMSR3 mismatch repair protein.

Applicants respectfully request withdrawal of the rejection.

The Provisional Doubling Patenting Rejection of Claims 16, 17, and 71

Claims 16, 17, and 71 are provisionally rejected under the judicially created doctrine of double patenting over claims 1-3 and 36 of copending application serial number 09/912,697. Applicants respectfully request that the rejection be held in abeyance *until* the claims are indicated otherwise to be in condition for allowance.

Respectfully submitted,



Date: June 23, 2005

Michelle Holmes-Son
Registration No. 47,660

Customer No. 22907

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Nucleotide Sequence of the *Salmonella typhimurium mutL* Gene Required for Mismatch Repair: Homology of MutL to HexB of *Streptococcus pneumoniae* and to PMS1 of the Yeast *Saccharomyces cerevisiae*

JOHN A. MANKOVICH, CHRISTINE A. McINTYRE, AND GRAHAM C. WALKER*

Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139

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The *mutL* gene of *Salmonella typhimurium* LT2 is required for *dam*-dependent methyl-directed DNA mismatch repair. We have cloned and sequenced the *mutL* gene of *S. typhimurium* LT2 and compared its sequence with those of the *hexB* gene product of the gram-positive bacterium *Streptococcus pneumoniae* and the *PMS1* gene product of the yeast *Saccharomyces cerevisiae*. MutL was found to be quite similar to the HexB mismatch repair protein of *S. pneumoniae* and to the mismatch repair protein PMS1 of the yeast *S. cerevisiae*. The significant similarities among these proteins were confined to their amino-terminal regions and suggest common evolution of the mismatch repair machinery in those organisms. The DNA sequence for *mutL* predicted a gene encoding a protein of 618 amino acid residues with a molecular weight of 67,761. The assignment of reading frame was confirmed by the construction of a chimeric protein consisting of the first 30 amino acids of LacZ fused to residues 53 through 618 of MutL. Interestingly, the presence of excess amounts of this fusion protein in wild-type *mutL*$^+$ cells resulted in a *trans*-dominant effect causing the cell to exhibit a high spontaneous mutation frequency.

Accurate duplication of chromosomes is of great importance if a cell line is to guarantee the proper transfer of genetic information from generation to generation. To this end, cells have evolved a number of repair systems that ensure a high level of replication fidelity. One such system (reviewed in references 2, 21, and 30) involves the correction of certain mismatched base pairs that have been missed by the proofreading elements of the DNA polymerase replication complex. In *Salmonella typhimurium* and *Escherichia coli*, this is accomplished by methyl-directed DNA mismatch repair and is thought to be directed by the transient undermethylation of adenine in the four-base sequence -GATC- of newly replicated DNA. The adenine in the sequence is methylated in the N^6 position by the action of the *dam* methylase (DNA adenine methyltransferase) (10, 15). This *dam*-dependent mismatch repair system requires the products of the *mutL*, *mutS*, *mutH*, and *uvrD* genes. Mutants that are defective in any of these genes exhibit an elevated spontaneous mutation frequency (4, 34). In vitro experiments have shown that hemimethylated -GATC- sites in DNA can direct the repair of specific mismatches in a purified *E. coli* mismatch repair reaction that requires MutL, MutS, MutH, and UvrD (16, 18; P. Modrich, personal communication). In addition, MutL and MutS are involved in very-short-patch repair, which also requires the product of the *dcm* gene, DNA cytosine methyltransferase (17). An in vitro cell-free repair system that requires *mutL*, *mutS*, and *dcm* gene products has also been developed for very-short-patch repair (B. Yashar and P. Modrich, personal communication).

Functionally similar repair systems have also been described for the gram-positive organism *Streptococcus pneumoniae* and for the yeast *Saccharomyces cerevisiae*. Mutants in the *hexA* or *hexB* gene of *S. pneumoniae* exhibit an elevated spontaneous mutation frequency (14, 26, 38). The same is true for the *pms1-1* and *pms1-2* mutants of the yeast *S. cerevisiae* (40). However, these repair systems appear to differ from methyl-directed repair in the method for daughter strand recognition. In *S. pneumoniae*, strand recognition is thought to be directed by breaks in the strand that is to be repaired (3, 14). In *S. cerevisiae*, methylation is unlikely to play a role for mismatch repair, since its DNA has neither adenine nor cytosine methylation (11, 28).

Previously, we have shown that the *mutL*, *mutS*, *mutH*, and *uvrD* genes of *S. typhimurium* are functionally analogous to the corresponding *E. coli* genes in that the cloned genes from *S. typhimurium* can fully complement the corresponding *E. coli* mutants both in vivo and in vitro (18, 23–25; J. Mankovich and G. Walker, unpublished data). We have sequenced the *mutS* gene of *S. typhimurium* (9), and the *mutS* gene of *E. coli* has recently been found to be approximately 94% homologous to the *mutS* gene of *S. typhimurium* at the amino acid sequence level (Ken Stacey, personal communication). Furthermore, we and Priebe et al. have found that MutS of *S. typhimurium* and HexA of *S. pneumoniae* exhibit approximately 38% overall identity when the amino acid sequences are optimally aligned (9, 27). However, these similarities are not distributed evenly throughout the proteins but are grouped in regions with homologies as high as 60%. This finding led us to predict the existence of evolutionarily related repair proteins in other widely divergent organisms, including eucaryotes (9). It is of interest to note that MutL and MutS are involved in a methylation-directed repair process, whereas HexA and HexB functions are independent of the methylation state of the DNA.

A number of in vitro biochemical activities associated with mismatch repair have been determined for the MutS (23, 36), MutH (39), and UvrD (helicase II) (12, 22, 37) proteins. At

* Corresponding author.

present, the precise role of MutL in the process of mismatch repair is unknown. In this paper, we report the nucleotide sequence for the *mutL* gene of *S. typhimurium* LT2 and show that the predicted amino acid sequence contains regions of homology to the mismatch repair proteins HexB and PMS1 of *S. pneumoniae* and *S. cerevisiae*, respectively.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and plasmids. All work with the *S. typhimurium mutL* gene was performed in *E. coli*, as the *mutL* gene from *S. typhimurium* is known to fully complement the *E. coli* gene (23). GW5100 (*E. coli* JM103 [P1 cured]) was used for α complementation of LacZ as well as for production of single-stranded and double-stranded templates for DNA sequencing. *E. coli* AB1157 (*trpA46 mutL*$^+$) and its isogenic *mutL218*::Tn*10* derivative were obtained from R. G. Fowler (1) and were used for *mutL* complementation studies. CSR603 (*recA1 uvrA6 phr-1*) was used in the maxicell experiments (32).

Plasmid pGW1842 (23) was used as the source for the subcloning of the *S. typhimurium mutL* gene. Vectors pBluescript KS(+) and SK(+) (Stratagene, La Jolla, Calif.) were used as vectors for cloning and sequencing *mutL*. Interference-resistant helper phage R408 (Stratagene) was used for single-stranded DNA rescue.

Media. M9, LB, and 2xYT media have been previously described (20). Antibiotics were used in the following concentrations (micrograms per milliliter): ampicillin, 100; tetracycline, 10; nalidixic acid, 40; and rifampin, 100.

In vivo complementation assay. Complementation of *mutL218*::Tn*10* by plasmids carrying various DNA fragments was determined by using a qualitative plate assay. Samples of 100 μl from overnight LB broth cultures were plated on nalidixic acid and rifampin plates. Complementation of the *mutL218*::Tn*10* allele by the plasmid resulted in fewer than 5 antibiotic-resistant colonies per plate, whereas failure to complement resulted in more than 100 colonies per plate.

To determine mutation frequencies for specific clones, single colonies were picked and grown in LB broth for 24 h. The cultures were diluted in saline, and approximately 100 to 1,000 cells were inoculated into fresh LB broth, followed by continued incubation for 24 h. The cells were then concentrated 10-fold in saline, and dilutions were plated on selective medium to determine mutation frequency.

DNA manipulations. Standard methods for analysis of DNA, such as restriction analysis, T4 DNA ligation, blunting of overhanging single-stranded ends, agarose gel electrophoresis, transformation, and plasmid and phage DNA purifications, were performed as described by Maniatis et al. (19).

DNA sequencing. A series of nested deletion mutants was constructed by using the exonuclease III-mung bean nuclease digestion protocol from Stratagene. This allowed isolation of clones containing sequential deletions from a fixed point in the polylinker of the vector to various points in the *mutL* gene. Any regions of the *mutL* gene that were not readily accessible for sequencing by the exonuclease III-mung bean nuclease treatment were made so by the removal of specific restriction fragments or by the use of synthetic oligonucleotides to prime chain elongation at specific sites.

Single-stranded and double-stranded DNA sequencing was carried out by the dideoxy-chain termination technique of Sanger et al. (33), using modified T7 DNA polymerase. Analysis of DNA sequence data was carried out using University of Wisconsin Genetics Computer Group software (5).

Construction and characterization of fusions. Previous restriction mapping of the 2.8-kilobase fragment of pGW1842 placed a unique *Hin*dIII site near the predicted amino terminus of the MutL protein (23). By using this restriction site and the *Bam*HI site in the polylinker of the vector, deletions of the amino terminus of MutL were made such that the promoter and amino terminus of the α-complementing fragment of β-galactosidase from the vector was fused to the *mutL* gene in all three reading frames.

The reading frame I fusion (pGW3403) was prepared by digesting pGW3402 with *Bam*HI, followed by filling in the 5′-overhanging single-stranded DNA with DNA polymerase I large (Klenow) fragment. This DNA was then further digested with *Hin*dIII, followed by removal of the 5′-overhanging single-stranded DNA with mung bean nuclease. The reading frame II fusion (pGW3404) was prepared by digesting pGW3402 with both *Bam*HI and *Hin*dIII, followed by removal of both 5′-overhanging ends by mung bean nuclease digestion. The reading frame III fusion (pGW3405) was prepared much the same as the reading frame II fusion except that both 5′-overhanging ends were filled in with DNA polymerase I large (Klenow) fragment. Each blunt-ended DNA mix was then ligated and transformed into a *mutL218*::Tn*10* strain. Representative clones from each transformation mix that had deleted the *Bam*HI-*Hin*dIII fragment were chosen for further study.

Identification of plasmid-encoded proteins. Proteins encoded by the *mutL*$^+$ plasmid (pGW3402) and the reading frame fusion plasmids were analyzed by the maxicell method (32).

RESULTS

Localization of the *mutL* gene. The *S. typhimurium mutL* gene was subcloned from plasmid pGW1842 (23) by insertion of the 2.8-kilobase *Sal*I-*Sma*I restriction fragment into the polylinker region of pBluescript KS(+) and SK(+) sequencing vectors. Insertion of the *mutL* fragment resulted in the *mutL* gene being placed in both orientations relative to the markers on the vector. Plasmids carrying the *mutL*-containing restriction fragment in either orientation were found to complement a *mutL218*::Tn*10* strain, causing reduction of the high spontaneous mutation rate to a wild-type level (data not shown).

To localize the *mutL* gene more precisely, a series of deletion mutants from one end of the cloned 2.8-kilobase fragment was constructed as described in Materials and Methods. These mutants were characterized with respect to the sizes of their deletions and the ability to complement a *mutL218*::Tn*10* strain (Fig. 1A). A subset of these deletions was used for DNA sequence determination.

DNA sequencing. The nucleotide sequence of the *S. typhimurium* LT2 *mutL* gene was determined (Fig. 2). The sequence was derived from both strands of the DNA spanning the entire region that complemented *mutL* (Fig. 1A). A single large open reading frame corresponding to the appropriate size for MutL was found (Fig. 1B). This open reading frame was transcribed in the same direction as that predicted by Pang et al. on the basis of the sizes of truncated polypeptides produced by *mutL*::Tn*1000* insertions (23). Fusion protein data supporting this reading frame assignment are described below.

Verification of reading frame choice. Our assignment of the open reading frame was confirmed by analysis of gene

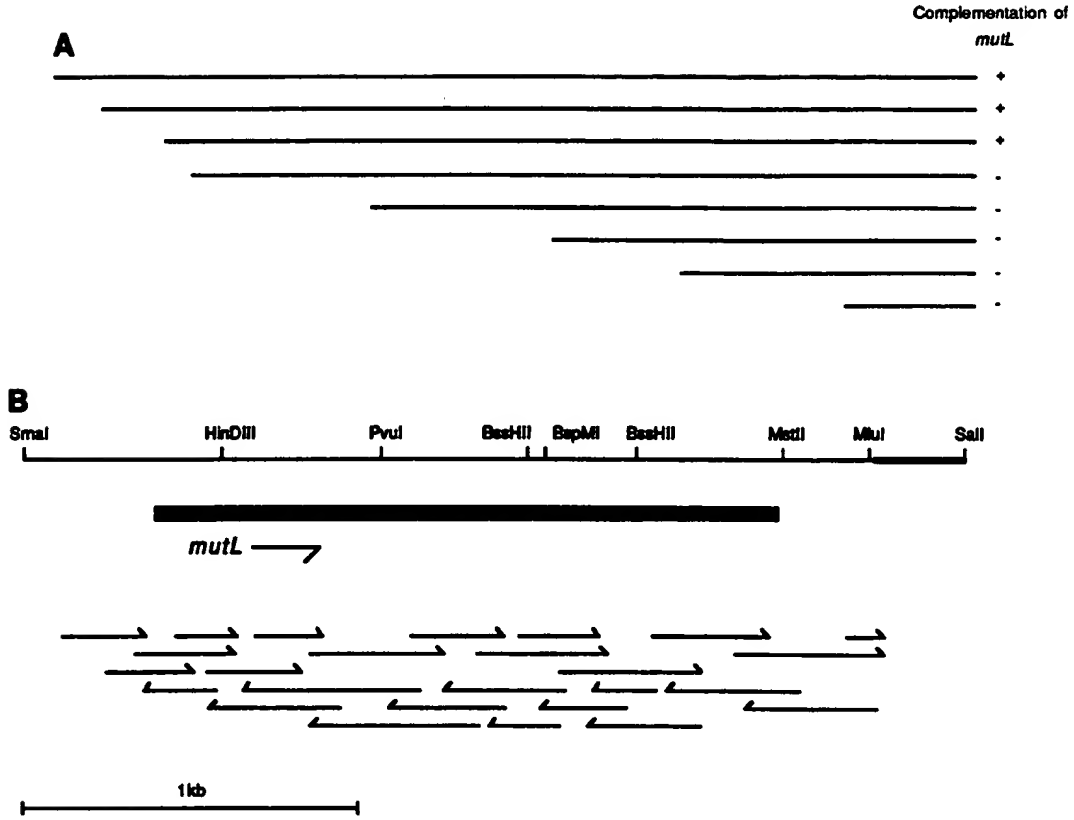

FIG. 1. Partial restriction map of the *mutL* gene region showing DNA sequencing strategy and location and predicted direction of transcription of the *mutL* gene. (A) DNA remaining after exonuclease III-mung bean nuclease digestion. + and −, Ability of each subclone to complement *mutL218*::Tn*10*. Horizontal arrows in panel B indicate the direction and length of the nucleotide sequence determined for each segment. The darkened area to the right of the *Mlu*I site indicates a segment of pBR322 DNA cloned along with the *mutL* gene from pGW1842. kb, Kilobase.

fusions in all three possible reading frames constructed by replacement of the amino terminus of MutL with that of the promoter and amino terminus of LacZ. These fusions were constructed by using the *Hin*dIII site in the *mutL* gene and the *Bam*HI site in the polylinker of the vector pSK(+) as described in Materials and Methods.

DNA sequence analysis was performed on three isolates to confirm each reading frame construction (Fig. 3). For reading frame II (pGW3404) and reading frame III (pGW3405), the DNA sequence results were as expected, given the enzyme analysis performed on the DNA fragments (see Materials and Methods). However, for reading frame I (pGW3403) the mung bean nuclease digestion of the *Hin*dIII terminus was incomplete, resulting in the removal of only one base instead of four. Since this gave a fusion to the intended reading frame, further screening for the predicted mutant was unnecessary.

The possibility that one of the reading frame fusions would produce a hybrid protein was tested by using the maxicell technique to label plasmid-encoded proteins. Figure 4 depicts the ^{35}S-labeled proteins encoded by the three reading frame fusion plasmids compared with that of the wild-type *mutL*$^+$ plasmid. In only one case (frame III, pGW3405) was there a ^{35}S-labeled protein band with an M_r appropriate for the fusion of LacZ with MutL (Fig. 4, lane C). This band was also found to correspond to a very abundant Coomassie-staining protein band that reacted to antibodies directed against MutL and did not appear in either of the other fusions (data not shown). Since this band was not observed in strains with only the chromosomal copy of *mutL*, the presence of the heavily stained protein band indicated that the fusion protein was in excess to the wild-type MutL protein. This fusion protein consists of the first 30 amino acids of LacZ fused to the predicted amino acid 53 of MutL. As a result, a glutamine residue corresponding to the large open reading frame shown translated in Fig. 2 was created at the fusion junction (Fig. 3).

Since less than 10% of the MutL protein had been deleted in construction of the frame III fusion, we investigated the possibility that this fusion could complement a *mutL* strain. The plasmid containing the frame III fusion (pGW3405) was transformed into a *mutL218*::Tn*10* strain, and the spontaneous mutation frequency was determined. The presence of the plasmid had no effect on the mutator phenotype of the *mutL218*::Tn*10* strain. However, elevated spontaneous mutation frequencies were observed when the frame III fusion plasmid (pGW3405) was transformed into a wild-type *mutL*$^+$ strain. This strain exhibited spontaneous mutation frequencies that were approximately 100-fold higher than that of wild-type cells containing either the vector or the cloned *mutL*$^+$ gene (Table 1). It is interesting that the high spontaneous mutation frequencies associated with the frame III fusion in wild-type *mutL*$^+$ cells were approximately five-

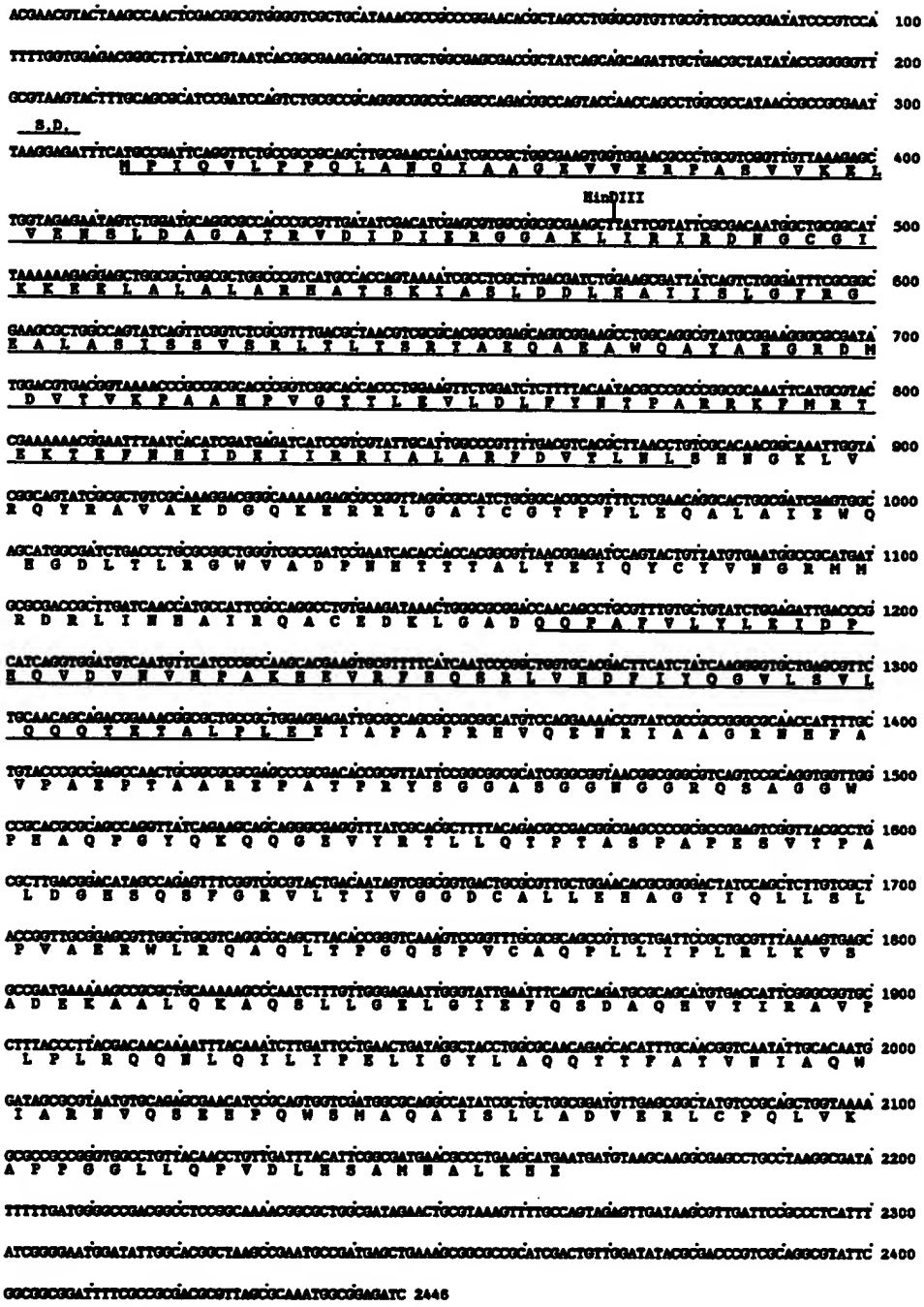

FIG. 2. Nucleotide sequence of *mutL* gene region from *S. typhimurium*. The derived amino acid sequence is indicated in one-letter code from positions 313 to 2169. The putative ribosome-binding site (S.D.) is indicated proximal to the start codon. The *Hin*dIII site used for generating the reading frame fusions is indicated at position 470. Regions of the amino acid sequence that have strongest similarities to HexB and PMS1 are underlined.

fold lower than those observed when the cells were *mutL218*::Tn*10*.

Comparison of *mutL* with mismatch repair genes of *S. pneumoniae* and the yeast *S. cerevisiae*. Previously, we and Priebe et al. reported that the mismatch repair genes *mutS* of *S. typhimurium* and *hexA* of *S. pneumoniae* contain regions of significant similarity at the amino acid sequence level (9, 27). The impetus for such a comparison was based in part on the similar sizes of the MutS and HexA proteins. Since MutL and HexB have similar M_rs (23, 26), a comparison of the predicted *mutL* and *hexB* amino acid sequences was carried out. The nucleotide sequence for the *hexB* gene of *S. pneumoniae* was determined (29), and a comparison of the predicted amino acid sequences of *mutL* and *hexB* revealed

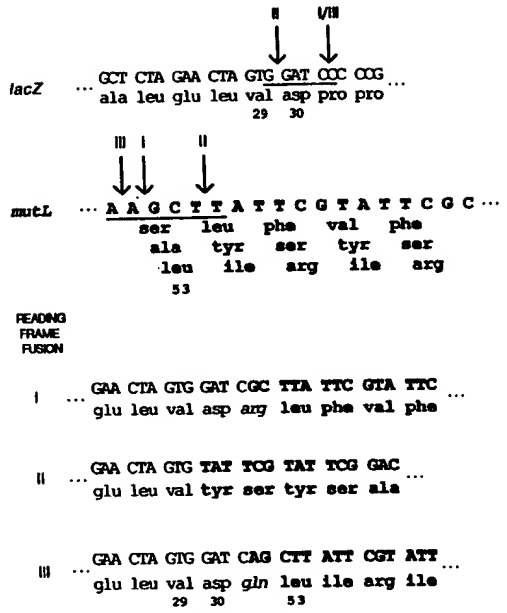

FIG. 3. Construction and partial sequences of reading frame fusions. Shown are DNA and predicted amino acid sequences around the *Bam*HI site in the polylinker of pBluescript SK(+) and the *Hin*dIII site of *mutL* and the resultant hybrid genes. Arrows indicate the point at which the fusion occurred at each restriction site. Numbers below the amino acids indicate numbers of amino acids from initial methionines of the intact molecules.

significant homologies between the two. The amino-terminal regions of the two putative DNA repair proteins had 99 of 189 (52%) identical amino acids (Fig. 5).

Since mutations in the *PMS1* gene of the yeast *S. cerevisiae* also result in mutator phenotypes (40), a parallel analysis was carried out to compare the deduced sequences of the *PMS1* (13) and *mutL* gene products. The amino termini of the proteins had 61 of 190 (32%) of the MutL residues being identical with PMS1, beginning at residue 1 of the predicted MutL protein and residue 31 of the PMS1 protein (Fig. 5). This homology was not as great as the homology seen between MutL and HexB. A similar comparison between HexB and PMS1 showed the same degree of homology as that seen between MutL and PMS1; however, not all of the residues that were identical between MutL and PMS1 corresponded to the same residues that were identical between HexB and PMS1. It is noteworthy that the same regions that were homologous between MutL and HexB were homologous between MutL and PMS1.

Comparison of all three proteins together showed that they had approximately 24% of their residues in common within the amino-terminal segment, with 45 of 190 residues being identical among them. These conserved residues were not distributed evenly throughout the amino-terminal sequences but rather were clustered in small segments of the homologous regions. Another segment of approximately 58 residues that contained areas of significant homology existed among the three proteins, starting at residue 284 for MutL, 279 for HexB, and 341 for PMS1 (Fig. 5). We found that 34%

TABLE 1. Mutation frequencies of *mutL* and wild-type cells carrying the frame III fusion plasmid

Plasmid	Mutation frequency (no. of mutants/10^8 viable cells)[a]			
	mutL218::Tn*10*		*mutL*$^+$	
	Nal^r	Rif^r	Nal^r	Rif^r
pSK(+) (vector)	113	109	<0.10	<0.13
pGW3402 (*mutL*$^+$)	<0.66	<1.28	<0.13	<0.53
pGW3405 (frame III)	97	340	27	38

[a] Determined as described in Materials and Methods. Values are averages of four trials. Nal^r, Spontaneous resistance to nalidixic acid; Rif^r, spontaneous resistance to rifampin.

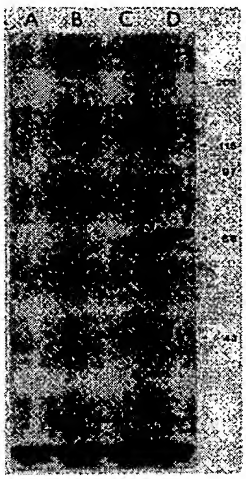

FIG. 4. Plasmid-encoded proteins produced by *lacZ-mutL* reading frame fusion plasmids and wild-type *mutL*$^+$ pGW3402. The maxicell technique was used to label plasmid-encoded proteins with [^{35}S]methionine. The labeled products were resolved by sodium dodecyl sulfate-electrophoresis through an 8% polyacrylamide gel and detected by fluorography. Lanes: A to C, reading frame fusions I (pGW3403), II (pGW3404), and III (pGW3405), respectively; D, wild-type *mutL*$^+$ (pGW3402) plasmid-encoded proteins. Molecular weights (in thousands) are shown on the right.

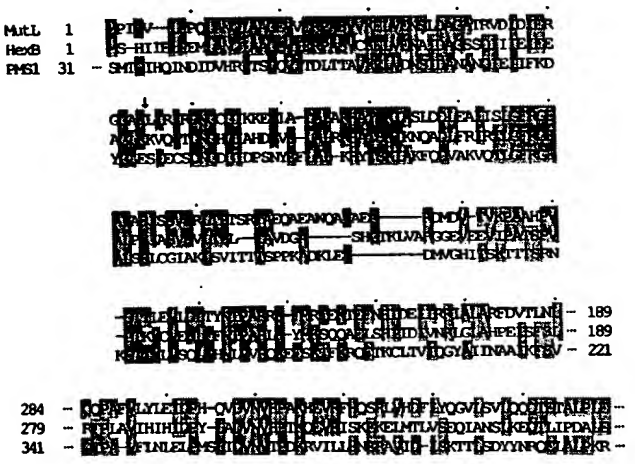

FIG. 5. Homologies of the deduced amino-terminal segments of the MutL, HexB, and PMS1 amino acid sequences. Numbers indicate the position of the corresponding amino acid for each sequence. Identical residues are shaded. Gaps in the sequences have been introduced to maximize the homologies. The point at which the first 30 amino acids of LacZ were fused to MutL is indicated by the vertical arrow.

(20 of 58) of the residues of MutL were identical to HexB residues and that 36% (21 of 58) were identical to PMS1 residues. The homology between HexB and PMS1 was somewhat less, 22% (13 of 58 residues). The similarities among all three proteins in this area were clustered in a single region of the segment where 41% (9 of 22) of the residues were identical.

A third region of homology between HexB and PMS1 was found in the carboxy termini. This region did not correspond to any portion of the MutL protein and is discussed in the accompanying papers (13, 29). Additional comparisons of these gene products with the mismatch repair proteins MutS, MutH, and UvrD were carried out, and no significant homologies were observed (6, 7, 9). Also, a computer search for protein and DNA sequence homologies with *mutL* by using current releases of the NBRF, EMBL, and GenBank data bases resulted in no significant homologies being found.

DISCUSSION

In this study, we present the complete nucleotide sequence for the *S. typhimurium mutL* gene, which is required for methyl-directed DNA mismatch repair. Given the amino acid sequence similarities observed between MutS of *S. typhimurium* and HexA of *S. pneumoniae*, we determined the nucleotide sequence for *mutL* and compared the predicted amino acid sequence with those of other relevant repair proteins now under investigation. On the basis of their similar molecular weights and the mutator phenotypes observed when these genes are defective, we compared MutL of *S. typhimurium* with HexB of *S. pneumoniae*. We also compared the *mutL* amino acid sequence with the predicted amino acid sequence of the *PMS1* gene of *S. cerevisiae*; mutations in *PMS1* also result in a mutator phenotype in yeast. The results of these comparisons revealed that the predicted MutL protein contains regions of significant amino acid sequence homology to the HexB protein of *S. pneumoniae*. Moreover, they show that these same regions have striking similarity to regions in the predicted *PMS1* gene product of the yeast *S. cerevisiae*. In addition, we have shown that an inactive fusion protein containing greater than 90% of the MutL protein expressed from a multicopy plasmid can inhibit mismatch repair in a *trans*-dominant fashion when present in wild-type cells.

The similarities found among MutL, HexB, and PMS1 and those described previously between MutS and HexA reinforce the notion that the DNA mismatch repair machinery evolved very early. There is greater similarity between MutL and HexB than there is between either MutL and PMS1 or HexB and PMS1. This finding may reflect the more recent divergence of the gram-positive and gram-negative bacteria (41). The fact that homologies exist between the procaryotic MutL and HexB proteins and the eucaryotic PMS1 protein suggests a common mode of action for these proteins in the repair of DNA mismatched base pairs. Also, amino acid sequence homologies may exist among various components of mismatch repair in a variety of other procaryotic and eucaryotic organisms.

The *mutL* DNA sequence of *S. typhimurium* predicts that the gene encodes a protein of 618 amino acid residues with a molecular weight of 67,761, which is in close agreement with data reported here and previously that *mutL* from *S. typhimurium* has an M_r of approximately 70,000 (23, 24), as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The choice of the correct reading frame for the *mutL* gene was made by creating translational fusions in all three reading frames and analyzing the fusions for the production of an appropriately sized peptide and for the exhibition of an appropriate phenotype. The reading frame choice was based on two observations: (i) only one of the reading frame fusion plasmids produces a plasmid-encoded protein with the correct M_r of the predicted fusion protein, and (ii) the presence of this fusion plasmid results in a partial mutator phenotype in a wild-type *mutL*$^+$ strain.

Not only does the reading frame fusion experiment allow the correct choice of reading frame for *mutL*, but it also gives insight as to possible interactions of MutL with the mismatch repair machinery. Greater than 90% of the MutL amino acid sequence is present in the fusion protein, which consists of the first 30 amino acids of LacZ fused to residues 53 through 618 of MutL. Given the large proportion of MutL in the fusion protein, it is likely that a subset of the MutL domains remains active and could account for the mutator phenotype observed when the fusion is present in wild-type cells.

There are two possible explanations for the apparent dominant negative phenotype observed when the LacZ-MutL fusion is present in wild-type cells. One possibility is that MutL is capable of regulating repair and that an overabundance of inactive or partially active MutL protein is sufficient to inhibit the production of a component or components of the mismatch repair machinery. This is unlikely given that no evidence for regulation of *mutL* has yet been found (23; P. Pang and G. Walker, unpublished data). The second possibility is that the *trans*-dominant effect is due to direct interaction of the LacZ-MutL protein with the less abundant active MutL (MutL dimers have been observed in vitro [8]) or with other components of the mismatch repair machinery. The higher-copy-number inactive fusion protein could be preventing the relatively few copies of active MutL from participating in the repair process. Consistent with this view is the observation that the presence of the fusion protein in *mutL*$^+$ cells results in spontaneous mutation frequencies that are less than that seen in a *mutL218*::Tn*10* strain. This finding suggests that repair is not completely eliminated but is merely reduced, possibly because of the intracellular dilution of active MutL by the more abundant LacZ-MutL fusion protein. Additional independent support comes from results of recent in vitro experiments suggesting an interaction between purified MutL protein and purified MutS protein on heteroduplexed DNA molecules (8).

The DNA sequence upstream of the predicted start codon AUG for *mutL* was examined for sequences that are associated with many *E. coli* promoters. A very good match for the ribosome-binding site, the Shine-Dalgarno sequence, was found just proximal to the predicted initiation codon AUG (35). However, no match to known consensus sequences for *E. coli* promoters (−35 and −10 regions) was obtained (31). The lack of a recognizable promoter in the sequenced region can be explained by results of recent experiments suggesting that the *mutL* gene of *E. coli* lies within a complex operon between an unknown gene encoding a protein of M_r 47,000 and the *miaA* gene (D. Connolly and M. Winkler, personal communication). It is possible that the transcriptional organization of *mutL* in *S. typhimurium* is similar, since we have previously observed that a gene encoding a 47,000-M_r protein is located upstream of the *mutL* gene in *S. typhimurium* (23). Furthermore, our sequencing revealed the presence of an uninterrupted reading frame that is present from the start of the sequenced region to a stop codon UAA just nine base pairs in front of the start codon for the *mutL* gene of *S. typhimurium*. This reading

frame is continuous from the beginning of the sequenced region but does not contain a start codon. Presumably, a start codon and promoter for this putative gene could be found by additional DNA sequence determination.

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Role of mutS and mutL genes in hypermutability and recombination in Staphylococcus aureus.

Prunier AL, Leclercq R.

Service de Microbiologie and EA 2128 Relations hote et microorganismes des epitheliums, Hopital Cote de Nacre, Universite de Caen, France.

The mutator phenotype has been linked in several bacterial genera to a defect in the methyl-mismatch repair system, in which the major components are MutS and MutL. This system is involved both in mismatch repair and in prevention of recombination between homeologous fragments in Escherichia coli and has been shown to play an important role in the adaptation of bacterial populations in changing and stressful environments. In this report we describe the molecular analysis of the mutS and mutL genes of Staphylococcus aureus. A genetic analysis of the mutSL region was performed in S. aureus RN4220. Reverse transcriptase PCR experiments confirmed the operon structure already reported in other gram-positive organisms. Insertional inactivation of mutS and mutL genes and complementation showed the role of both genes in hypermutability in this species. We also designed an in vitro model to study the role of MutS and MutL in homeologous recombination in S. aureus. For this purpose, we constructed a bank of S. aureus RN4220 and mutS and mutL mutants containing the integrative thermosensitive vector pBT1 in which fragments with various levels of identity (74% to 100%) to the S. aureus sodA gene were cloned. MutS and MutL proteins seemed to have a limited effect on the control of homeologous recombination. Sequence of mutS and mutL genes was analyzed in 11 hypermutable S. aureus clinical isolates. In four of five isolates with mutated or deleted mutS or mutL genes, a relationship between alterations and mutator phenotypes could be established by negative complementation of the mutS or mutL mutants.

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1: Mol Microbiol. 2002 May;44(3):877-87.

A hypermutator phenotype attenuates the virulence of Listeria monocytogenes in a mouse model.

Merino D, Reglier-Poupet H, Berche P, Charbit A; European Listeria Genome Consortium.

INSERM U-411, CHU Necker-Enfants Malades, 156 rue de Vaugirard, 75730 Paris Cedex 15, France.

The integrity of the genetic material of bacteria is guaranteed by a set of distinct repair mechanisms. The participation of these repair systems in bacterial pathogenicity has been addressed only recently. Here, we study for the first time the participation in virulence of the MutSL mismatch repair system of Listeria monocytogenes. The mutS and mutL genes, which are contiguous in the L. monocytogenes chromosome, were identified after in silico analysis. The deduced MutS shares 62% identity with MutS of Bacillus subtilis and 50% identity with HexA, its homologue in Streptococcus pneumoniae; MutL shares 59% identity with MutL of B. subtilis and 47% identity with HexB of S. pneumoniae. Functional analysis of the mutSL locus was studied by constructing a double knock-out mutant. We showed that the deletion DeltamutSL induces: (i) a 100- to 1000-fold increase in the spontaneous mutation rate; and (ii) a 10- to 15-fold increase in the frequency of transduction, thus demonstrating the role of mutSL of L. monocytogenes in both mismatch repair and homologous recombination. We found that the deletion DeltamutSL moderately affected bacterial virulence, with a 1-log increase in the lethal dose 50% (LD50) in the mouse. Strikingly, repeated passages of the mutant strain in mice reduced virulence further. Competition assays between wild-type and mutant strains showed that the deletion DeltamutSL reduced the capacity of L. monocytogenes to survive and multiply in mice. These results thus demonstrate that, for the intracellular pathogen L. monocytogenes, a hypermutator phenotype is more deleterious than profitable to its virulence.

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Bacillus subtilis mutS mutL operon: identification, nucleotide sequence and mutagenesis.

Ginetti F, Perego M, Albertini AM, Galizzi A.

Dipartimento di Genetica e Microbiologia, Universita degli Studi di Pavia, Italy.

The Bacillus subtilis mutS and mutL genes, involved in the DNA mismatch repair system, have been cloned and characterized. From sequence analysis the two genes appear to be organized in a single operon, located immediately downstream of the cotE gene (approximately 150 degrees on the genetic map). The deduced MutS protein is 49% identical to HexA and MutL is 46% identical to HexB of Streptococcus pneumoniae. Deletion of both mutS and mutL resulted in an increase in the frequency of spontaneous mutations and abolished the marker effect observed in transformation. The expression of the mut operon was studied with the use of a mutSL-lacZ transcriptional fusion. An increase in expression was observed during late exponential growth.

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